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Simultaneous determination of tiopronin and D-penicillamine in human urine by liquid chromatography with ultraviolet detection

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Abstract

D-Penicillamine and tiopronin are drugs widely used for the treatment of many diseases. Because of the relatively high frequency of side effects to these compounds, some of which are dose-related, drug monitoring in urine samples during treatment is advisable. In this paper, we describe a simple method for the determination of tiopronin and D-penicillamine in human urine. The method was based on derivatization with 2-chloro-1-methylquinolinium tetrafluoroborate followed by ion-pairing reversed-phase liquid chromatography separation and ultraviolet-absorbance detection. 2-*S*-quinolinium derivatives of thiols were detected at 355 nm. The derivatization was optimized in terms of pH and time of the reaction. Baseline separation was achieved on an analytical Zorbax SB C-18 (5 μm, 150 mm × 4.6 mm) column with a mobile phase consisting of pH 2.0 0.09 mol L^{-1} trichloroacetic acid buffer (component A) and acetonitrile (component B) pumped at 1.0 mL min^{-1}. Gradient elution was used: 0–4 min, 12% B; 4–8 min, 12–40% B; 8–12 min, 40–12% B. The D-penicillamine and tiopronin standards added to the urine show that the response of the detector is linear within the range studied, from 1 to 200 μmol L^{-1} urine. The imprecision ranges for tiopronin and D-penicillamine were within 1.61–8.24% and 2.92–10.60%, respectively. The analytical accuracy for determined compounds was from 97.24 to 109.39%. The lower limits of detection and quantitation were 0.5 μmol L^{-1} and 1.0 μmol L^{-1} urine, respectively. This method can be used for routine clinical monitoring of the title thiol-drugs. Cysteine can be measured concurrently, if needed.

Keywords: D-Penicillamine; Tiopronin; Cysteine; Liquid chromatography; Derivatization; Urine

1. Introduction

D-Penicillamine and tiopronin are pharmaceutically important thiol compounds. D-Penicillamine (2-amino-3-mercapto-3-methylbutanoic acid, β,β-dimethylcysteine, 3-mercaptovaline) is an unphysiological sulfur-containing amino acid that belongs to the aminothiol family with a hydrogen atom in the beta-carbon of cysteine replaced by the methyl group. It can exist in D and L enantiomeric forms; however only the D type is clinically useful due to excessive toxicity of the L type [1]. D-Penicillamine is a thiol drug used in the treatment of Wilson's disease, an autosomal recessive disorder of copper transport [2]. It is also used as antifibrotic agent to treat scleroderma [3] and as antirheumatic drug to treat patients with active rheumatoid arthritis [4].

Tiopronin (MPG, *N*-(2-mercaptopropionyl)-glycine, Thiola) is a sulphydryl compound with properties similar to those of D-penicillamine. MPG is a synthetic compound that acts as a potent free radical scavenger [5]. It has been shown to protect against ischemic/reperfusion-mediated injury [6] and could also be effective against radiation-induced damages, even at low doses [7]. Tiopronin is also used in the treatment of rheumatoid arthritis [8], hepatic diseases and as a mucolytic in respiratory disorders [5,8].

Both drugs are efficient antidotes to heavy metal poisoning [8,9] and have been frequently used for the treatment of cystinuria, an autosomal recessive genetic defect of the transepithelial transport of cystine and other dibasic amino acids in the kidney [10,11]. These drugs prevent the formation of cystine stones in kidney when there is too much cystine in the urine. Tiopronin and D-penicillamine transform cystine into a mixed disulfide, 50 times more soluble than cystine itself [10].

Both D-penicillamine and tiopronin are rapidly absorbed from the gastrointestinal tract and are rapidly oxidized to various

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disulphide forms. About 42% of a standard 1 g intravenous dose of D-penicillamine is excreted in the urine over 24 h; 25–50% of a similar oral dose is excreted in the urine over 24 h [12]. Urinary excretion of tiopronin after single oral dose (500 mg) was mainly confined to the first 6 h (74%) and was almost complete (98%) within 12 h [13].

Severe adverse reactions to oral thiol-drugs use have been described in subjects in which abrupt incremental dosing of the drugs were started. Both sulfhydryl agents, especially D-penicillamine, provoke frequent side-effects, including ageusia or dysgeusia rash, pemphigus, thrombocytopenia, myasthenia gravis, agranulocytosis, polymyositis, proteinuria, or hypersensitivity nephritic syndrome [1,10,14]. This suggests that monitoring the concentrations of these compounds in biological fluids over the course of therapy is warranted and consequently a number of different methods of quantitating these thiols have been described [15–24].

Methods used for quantitation of D-penicillamine include a capillary electrophoresis (CE) with laser-induced fluorescence [9,15] and high-performance liquid chromatography (HPLC) with chemiluminescence [16], fluorescence [17,18] or electrochemical [19] detection. Tiopronin has been determined by capillary electrophoresis [15] and HPLC with ultraviolet [20], mass spectrometric [21] and fluorescence detection [22–24]. However, only one method, described by Lochman et al. [15], offers a procedure for simultaneous determination of both drugs.

In this paper, we describe an HPLC method for the simple simultaneous analysis of D-penicillamine and tiopronin in human urine after derivatization with 2-chloro-1-methylquinolinium tetrafluoroborate (CMQT). The method relies on transformation of the determined compounds to their *S*-quinolinium derivatives, separation by ion-pairing reversed-phase chromatography, and UV-absorbance detection and quantitation.

2. Experimental

2.1. Chemicals and reagents

2-Chloro-1-methylquinolinium tetrafluoroborate (CMQT) – a derivatization reagent – was synthesized in our laboratory as described earlier [25]. Perchloric acid (PCA), hydrochloric acid (HCl), sodium hydroxide (NaOH), sodium hydrogen phosphate heptahydrate ($Na_2HPO_4{\cdot}7H_2O$) and sodium dihydrogen phosphate dihydrate ($NaH_2PO_4{\cdot}2H_2O$) were from J.T. Baker (Deventer, Netherlands). Trichloroacetic acid (TCA), sodium borohydride ($NaBH_4$) and lithium hydroxide monohydrate ($LiOH{\cdot}H_2O$) were from Merck (Darmstadt, Germany). L-Cysteine was from Reanal (Budapest, Hungary), HPLC-grade acetonitrile was from Labscan (Dublin, Ireland). Dimethyl sulfoxide (DMSO) and *n*-octanol were obtained from Pierce (Rockford, IL, USA). D-Penicillamine and tiopronin were received from Fluka (Buchs, Switzerland).

For derivatization a 0.1 mol L^{-1} water solution of CMQT was used. Stock standard solutions of tiopronin, D-penicillamine and L-cysteine calibrator (0.1 mol L^{-1}) were prepared by dissolving an appropriate amounts of the compounds in 0.1 mol L^{-1} HCl. Solution of $NaBH_4$ (6 mol L^{-1}) was prepared by dissolving appropriate amount of the compound in 0.1 mol L^{-1} sodium hydroxide and diluted 2:1 with dimethyl sulfoxide. To prepare phosphate buffer (0.2 mol L^{-1}) appropriate quantities of sodium hydrogen phosphate heptahydrate ($Na_2HPO_4{\cdot}7H_2O$) and sodium dihydrogen phosphate dihydrate ($NaH_2PO_4{\cdot}2H_2O$) solutions were mixed. TCA buffer was prepared using 0.09 mol L^{-1} trichloroacetic acid and adjusted to the desired pH (2.0) with lithium hydroxide monohydrate (0.09 mol L^{-1}). The pH of the buffers was adjusted by potentiometric titrations. All chemicals were of analytical or HPLC grade and all solutions were prepared from deionized water. All the stock solutions were stored in the refrigerator at +4 °C.

2.2. Instrumentation

HPLC analysis was carried out using a Hewlett-Packard 1100 system (Waldbronn, Germany) equipped with quaternary pump, an autosampler, thermostated column compartment, vacuum degasser and diode-array detector. The system was controlled by HP ChemStation software. The separations were accomplished with a Zorbax SB C-18 (5 μm, 150 mm × 4.6 mm) analytical column (Agilent Technologies, Waldbronn, Germany). Water for solutions was prepared with a MILLI-Q-RG system (Millipore, Vienna, Austria). For pH measurement a HI 221 (Hanna Instruments, Woonsocket, RI, USA) pH meter was used.

2.3. Cleavage of the disulfide bonds

A portion of urine thiols occur in the disulfide forms (symmetrical and mixed) and in order to render them accessible to the derivatization reagent disulfide bonds must be cleaved with a reducing reagent to liberate free thiols. For this purpose sodium borohydride [26] was used.

2.4. Sample preparation

To freshly collected tiopronin- and D-penicillamine-containing urine (100 μL) were added 50 μL of *n*-octanol, 100 μL of $NaBH_4$ (6 mol L^{-1}) in 0.1 mol L^{-1} NaOH diluted with DMSO in the ratio 2:1 and 60 μL of HCl (3 mol L^{-1}). After 1 min 50 μL of hydrochloric acid (3 mol L^{-1}) was added in order to decompose excess of sodium borohydride (pH ≈ 7.5, indicator paper). Next, 100 μL of 0.2 mol L^{-1} phosphate buffer (pH 7.5) and 30 μL of CMQT reagent (0.1 mol L^{-1}) were added. The mixture was vortex-mixed and incubated at room temperature for 5 min. After the reaction, the mixture was acidified with 100 μL of PCA (3 mol L^{-1}) followed by centrifugation (12,000 × *g*, 5 min). A 20 μL of the supernatant was injected into the HPLC system.

2.5. Chromatographic conditions

Sample was injected using an autosampler into a 4.6 mm × 150 mm, 5 μm particle, Zorbax SB C-18 column (Agilent Technologies, Waldbronn, Germany). The mobile phase consisted of 0.09 mol L^{-1} TCA buffer, adjusted to pH 2.0 with lithium hydroxide monohydrate solution at the same concen-